Kinetics and regulation of de novo centriole assembly: Implications for the mechanism of centriole duplication

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Background: Centriole duplication is a key step in the cell cycle whose mechanism is completely unknown. Why new centrioles always form next to preexisting ones is a fundamental question. The simplest model is that preexisting centrioles nucleate the assembly of new centrioles, and that although centrioles can in some cases form de novo without this nucleation, the de novo assembly mechanism should be too slow to compete with normal duplication in order to maintain fidelity of centriole duplication.

Results: We have measured the rate of de novo centriole assembly in vegetatively dividing cells that normally always contain centrioles. By using mutants of *Chlamydomonas* that are defective in centriole segregation, we obtained viable centrioleless cells that continue to divide, and find that within a single generation, 50% of these cells reacquire new centrioles by de novo assembly. This suggests that the rate of de novo assembly is approximately half the rate of templated duplication. A mutation in the VFL3 gene causes a complete loss of the templated assembly pathway without eliminating de novo assembly. A mutation in the centrin gene also reduced the rate of templated assembly.

Conclusions: These results suggest that there are two pathways for centriole assembly, namely a templated pathway that requires preexisting centrioles to nucleate new centriole assembly, and a de novo assembly pathway that is normally turned off when centrioles are present.

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Background

One of the longest-standing mysteries in cell biology is the mechanism by which centrioles duplicate. Centrioles are cylindrical arrays of triplet microtubules that organize the centrosome [1]. Cells with too few or too many centrioles can form aberrant spindles, leading to chromosome segregation errors. It is therefore critical that the number of centrioles doubles exactly once per cell division. This doubling occurs by a remarkable process in which a single new centriole forms adjacent to, and at right angles with, each preexisting centriole. But why do new centrioles only form next to old ones? The geometry of centriole duplication suggests that preexisting centrioles somehow act as a template or catalyze the assembly of new centrioles, and this could account for why new centrioles only form next to old ones.

Consistent with the idea that centrioles are needed to form new centrioles, parthenogenetic development in *Xenopus* can only occur when a structurally intact centriole is provided to the egg [2]. Likewise, if unfertilized *Sciara* eggs are induced to develop parthenogenetically, they undergo multiple rounds of aberrant mitosis but never generate centrioles [3].

There are other cases where centrioles can self-assemble de novo, however. By de novo assembly, we mean the assembly of new centrioles independent of preexisting centrioles. Examples of de novo centriole assembly include parthenogenetic development of sea urchin and *Spisula* [4, 5], gametogenesis in lower plants and fungi [6, 7], and differentiation of protists such as *Naegleria* and *Oxytricha* [8, 9]. These demonstrations of de novo centriole assembly in specialized cell types have failed to dislodge the concept that centrioles nucleate new centriole assembly for the simple reason that they do not address the question of why, in ordinary cells that contain centrioles, centriole assembly only occurs adjacent to preexisting centrioles.

Moreover, when centrioles were physically removed from fertilized sea urchin embryos by cell fragmentation [10] or from mammalian tissue culture cells by microsurgery [11], centrioles did not regenerate de novo in the nucleated cell fragments that remained. This suggested that, in contrast to the parthenogenetic systems described above, centriole assembly in somatic cells might strictly require preexisting centrioles.

How then can we reconcile the fact that centrioles *can* form de novo in certain cases with the fact that centrioles *do not* form de novo in ordinary cells? A way to resolve

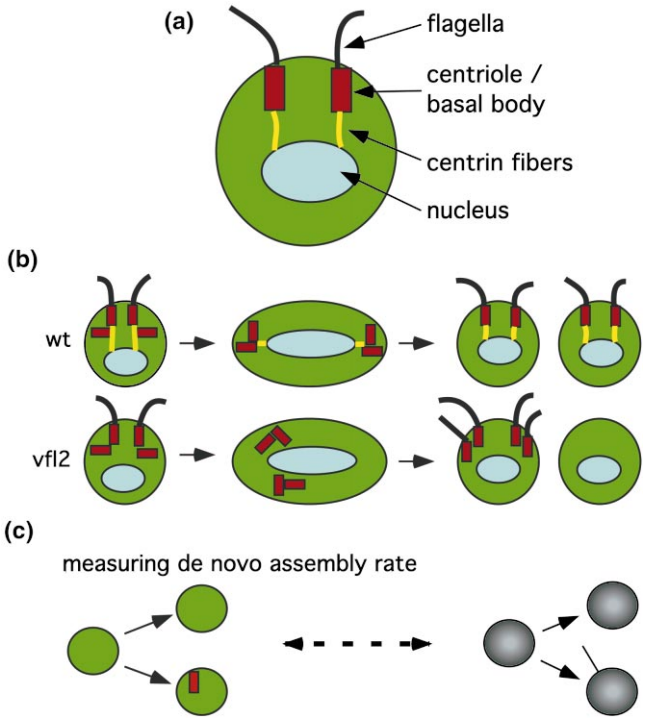
this apparent contradiction comes from the fact that in all published cases of de novo assembly, the cell types in question are developmentally primed specifically to generate centrioles de novo, and therefore contain massive reserves of centriole precursor proteins. For example, unfertilized oocytes are tightly packed with enough precursor proteins to assemble thousands of centrioles [12]. The ability of centrioles to form in the presence of such huge stockpiles of precursors is hardly surprising. De novo assembly might be much less efficient, or even impossible, in normal dividing cells that lack such large quantities of precursors.

Thus, a simple model can reconcile the obvious high fidelity of centriole duplication in normal cells with the ability of centrioles to assemble de novo in special cell types: in cells that ordinarily contain centrioles to start with, de novo centriole assembly is either impossible, or else occurs at such a slow rate that it cannot compete with assembly that is templated by preexisting centrioles.

The key to testing this model is to measure the rate of de novo centriole assembly in cells that normally contain centrioles, to see whether it really is too inefficient to compete with templated assembly. Previous experiments in which centrioles were removed from somatic cells [10, 11] did not result in de novo centriole assembly. The cell fragments did not divide after microsurgery, however, suggesting that the lack of de novo assembly could be due either to damage caused by microsurgery or to subsequent perturbation of cell division. We have therefore developed a gentler genetic strategy for measuring the rate of de novo centriole assembly in centrioleless cells that continue to divide. This approach exploits the genetics of *Chlamydomonas reinhardtii*, a unicellular haploid green alga that has many of the same genetic advantages as yeast [13], but unlike yeast, has centrioles indistinguishable from those of higher eukaryotes.

Our strategy takes advantage of the *vfl2* mutation of *Chlamydomonas* [14], a mutation of the centrin gene in which centriole segregation is defective. Due to this defect, a fraction of daughter cells fails to inherit any centrioles after cell division. Such centrioleless cells continue to divide, and are able to regenerate centrioles de novo, demonstrating that de novo centriole assembly is not limited to specialized reproductive cells but can occur in vegetative cells that normally contain centrioles. Surprisingly, the results show that centrioles can form de novo at a very high rate, with roughly half of all centrioleless cells forming a new centriole within one generation. This argues against the simple model above, and suggests a negative regulatory pathway must exist to keep de novo centriole assembly turned off when centrioles are present.

Figure 1



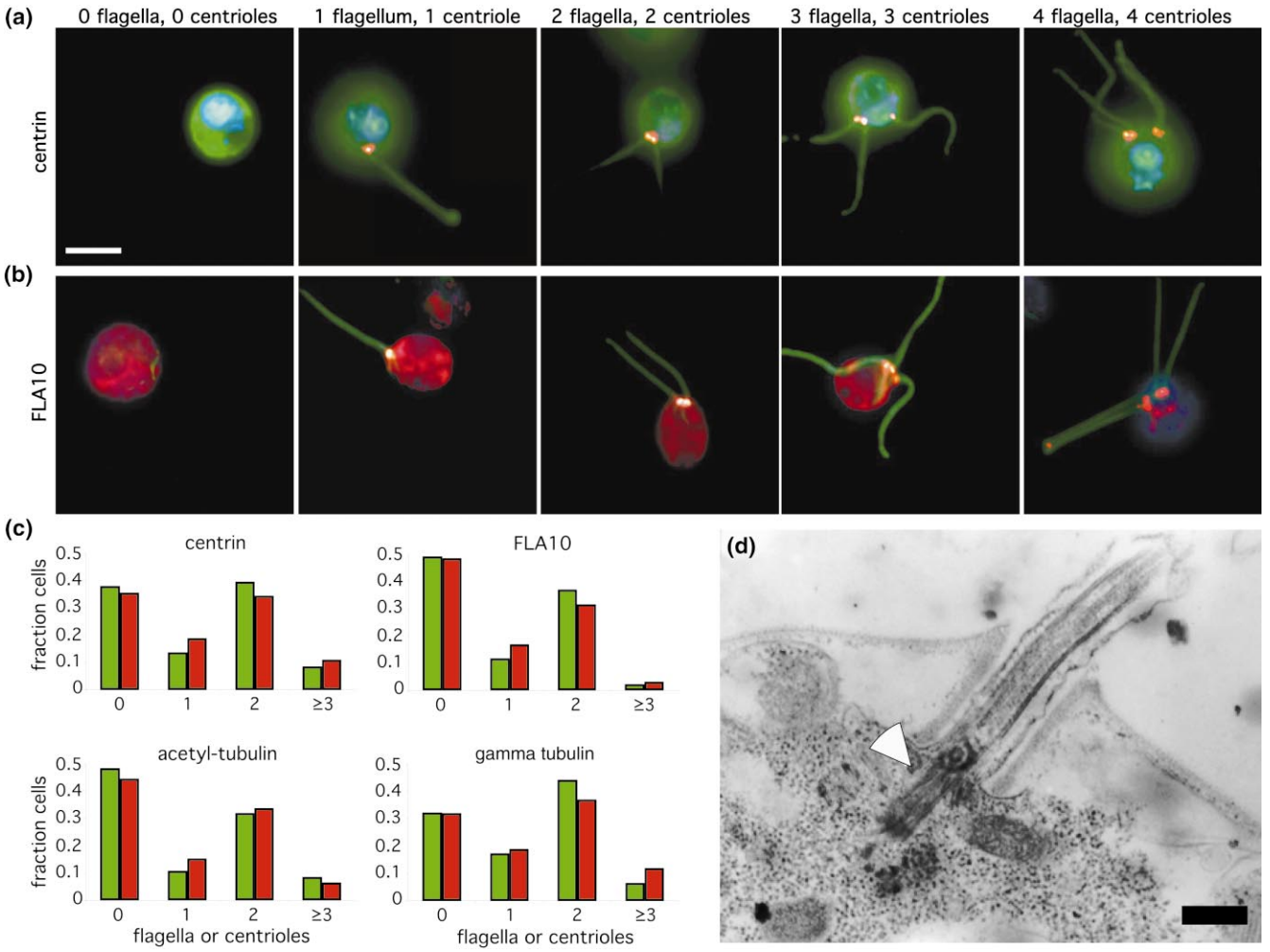
Strategy to measure de novo and templated centriole assembly. **(a)** Structure of a *Chlamydomonas* cell. Centrioles nucleate flagella in interphase and are physically attached to the nucleus by centrin fibers. **(b)** *vfl2* mutants lack centrin fibers. Because centrioles are no longer attached to the nucleus, they segregate randomly in mitosis, leading in some cases to the production of centrioleless cells. **(c)** Strategy to measure de novo centriole assembly. Centriole segregation mutants produce cells that are lacking centrioles. The recovery of centrioles indicates de novo assembly. Visualization of flagella can be used to deduce the underlying centriole inheritance pedigree in living cells, allowing the de novo assembly rate to be measured.

Results

Strategy for measuring templated and de novo centriole assembly

To measure de novo assembly, we recognize that centrioles in *Chlamydomonas* give rise to flagella during interphase (Figure 1a), which provides a uniquely efficient way to determine centriole content in living cells simply by counting flagella. *vfl2* mutants, unlike wild-type cells that segregate exactly two new centrioles to each progeny cell, segregate centrioles randomly to their daughters. This is because the *vfl2* mutation, a mutation in the centrin gene, causes reduced centrin levels and a failure of centrin to assemble into the nucleus–basal body–connecting fibers (Figure 1b) [14, 15, 16]. When *vfl2* mutant cells divide, some daughter cells end up without centrioles. By following individual centrioleless cells as they divide (Figure 1c), we can measure the rate at which they or their progeny recover centrioles de novo. This approach for measuring the de novo centriole assembly rate in dividing cells is conceptually similar to the classic

Figure 2



Centriole number equals flagellar number in *vfl2* mutants. **(a)** Examples of *vfl2* cells containing zero to four centrioles detected by centrin localization. Centrin (red) flagella (green) detected by antibodies to tubulin. In each case, the number of centrioles (red) matches the number of flagella (green). The scale bar represents 5 μm. **(b)** Examples of *vfl2* cells containing 0–4 centrioles detected by Fla10 kinesin localization. FLA10 (red) flagella (green) detected by antibodies to acetylated tubulin. The scale bar represents 5 μm. **(c)** Distribution of centrioles per cell as judged by centrin, FLA10, acetyltubulin, or γ-tubulin (red bars), compared with the distribution of flagella per cell (green bars). **(d)** Electron micrograph of a *vfl2* cell showing the longitudinal section of a centriole (arrow) with an associated flagellum. The scale bar represents 0.25 μm.

fluctuation analysis developed by Luria and Delbrück [17] for measuring the spontaneous mutation rate in dividing bacteria.

Monitoring centriole number in living *vfl2* cells

The strategy in Figure 1 is based on using flagella to track centriole assembly and inheritance. In wild-type cells, all centrioles always form flagella during interphase, but because our strategy is based on a mutant, it was necessary to verify that in *vfl2* cells, all centrioles are functional as basal bodies to make flagella. In other words, we must confirm that the number of flagella per cell equals the number of centrioles. The equivalence of centriole number and flagellar number in *vfl2* mutant cells was confirmed by both immunofluorescence and electron microscopy (Figure 2).

The number of centrioles in individual cells was determined using immunofluorescence with several centriole-specific antibodies. Centrin, a core component of the centriole, is frequently used as a specific marker to visualize centrioles throughout the cell cycle in a wide range of species [18–22]. Centrin also localizes to centrioles during the early stages of de novo assembly in ferns and *Naegleria* [23, 24], and has been shown to lose its localization from cells in which centrioles degenerate during development

[24, 25]. As seen in Figure 2a, *vfl2* cells containing zero, one, two, three, or four centrioles as judged by centrin immunofluorescence contain zero, one, two, three, or four flagella, respectively. Significantly, flagellaless cells do not contain centrioles. We note that although *vfl2* mutants have a defect in the centrin gene, the allele used in this study is a point mutation that still allows production of some centrin protein, albeit at reduced levels. This mutant centrin remains associated with the centriole [26].

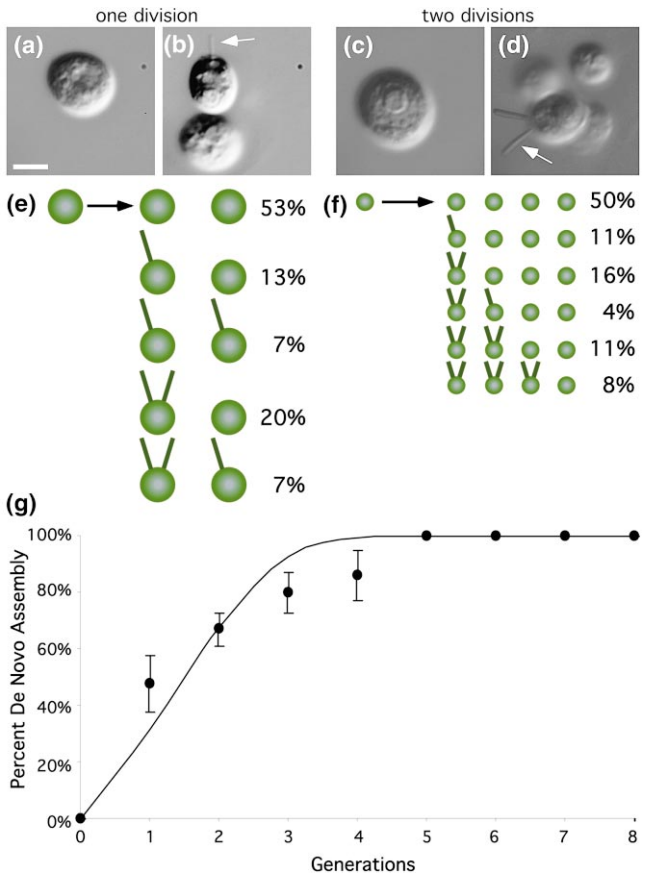
A similar correspondence between centrioles and flagella was seen using antibodies to the centriole-associated kinesin, Fla10 (Figure 2b). Fla10 is centriole-associated throughout the cell cycle [27] in a centriole-dependent manner [28]. Moreover, Fla10 localizes properly even to immature centrioles in *uni1* mutants (data not shown). Because the presence of a centriole is necessary and sufficient for Fla10 localization, the correlation of Fla10 foci with flagella in *vfl2* cells supports the equivalence of centriole and flagellar number.

The equivalence of centriole and flagellar number was tested statistically (Figure 2c). Antibodies against five different centriole-associated proteins, centrin, Fla10, acetylated tubulin [29], γ-tubulin [30], and p210 [31] all gave the same result, namely that the distribution of centrioles per cell as judged by immunofluorescence was identical to the distribution of flagella per cell as judged by phase contrast microscopy ($X^2 = 5.8$, $0.1 < P < 0.25$ for the Fla10 data). In all cases, a significant fraction of cells lacked centriole staining. A sixth antibody to the centriole protein Bap95 [32] also indicated that a comparable fraction of *vfl2* cells lacked centrioles (data not shown).

Importantly, all six centriole-specific proteins are known to be centriole-associated throughout the cell cycle including both interphase and mitosis [16, 20, 27]. Significantly, both centrin and p210 have been shown by immunoelectron microscopy [20] to associate with procentrioles during the initial stages of centriole assembly. Therefore, if flagellaless *vfl2* cells contained any immature procentrioles, they would have been detected.

To further confirm the equivalence of centriole number and flagellar number, electron microscopy was used to visualize the centriole directly. This analysis was prompted by the fact that a different *Chlamydomonas* centriole segregation mutant, *vfl3*, was previously shown by electron microscopy to contain many centrioles that were not associated with the cell surface and that did not give rise to flagella [33]. In contrast, it was reported that all centrioles seen in *vfl2* cells by electron microscopy were associated with flagella [33], although specific data were not presented. We found that in *vfl2* mutants, 100% of centrioles (n = 12) seen in longitudinal view by thin-section electron micrographs are associated with the cell surface at the base of flagella (for example, see Figure 2d).

Figure 3



Single-cell pedigree analysis reveals de novo centriole assembly. **(a–d)** DIC images of single nonflagellated cells and their flagellated progeny. **(a,b)** A cell that underwent a single division. The arrow indicates a flagellum. The scale bar represents 5 μm. **(c,d)** A cell that underwent two divisions to yield four progeny cells at least one of which is flagellated (arrow). **(e)** Pedigree results for cells undergoing a single division. For each outcome, the fraction of observed cases is given (based on the pedigrees of 15 cells). **(f)** Pedigree results for cells undergoing two rounds of division (n = 26). **(g)** The kinetics of de novo centriole assembly in dividing *vfl2* cells were obtained by single-cell pedigree analysis.

Single-cell pedigree analysis of centriole inheritance

Having shown that flagellar number equals centriole number in *vfl2* cells, we can now use flagella to monitor centriole number in dividing cells. In the most direct approach, living *vfl2* cells were embedded in a thin agarose pad, which allowed living cells to be imaged over several generations (Figure 3a–d). The embedded cell technique was impractical for more than two generations because the progeny formed a microcolony in which the flagella of individual cells were hard to see. A second method of pedigree analysis was therefore used to extend the

time course by suspending single cells in microtiter wells and inferring flagellar number by observing cell motility after varying periods of growth (see Materials and methods) [34].

We used this single-cell pedigree analysis to measure the kinetics of de novo centriole synthesis by looking for recovery of flagella in the progeny of nonflagellated (and thus centrioleless) cells. As shown in Figure 3e, after a single generation, almost 50% of flagellaless cells have produced flagellated progeny (which must, therefore, contain centrioles). As plotted in Figure 3g, within five generations, 100% of cells produce flagellated progeny. This suggests that de novo centriole assembly occurs efficiently in these cells. Comparing a de novo assembly rate of 0.5 new centrioles per cell per generation with the normal templated assembly rate of one new centriole per centriole per generation, we find that the presence of a centriole only increases the rate of new centriole assembly by a factor of two. We note that these rates are measured in the *vfl2* mutant background, which, as shown below, causes a partial defect in normal centriole duplication. Therefore, the actual rate of de novo assembly might be even higher than that measured here.

Cell cycle regulation of de novo assembly

Is de novo assembly under the same cell cycle control as normal duplication? To answer this question, we first determined the cell cycle dependence of normal centriole duplication in *Chlamydomonas*. As seen in Figure 4a, cell cycle mutants arrested in G1 prior to the commitment to divide [35, 36], as well as wild-type cells arrested in G1 by growth in the dark [37], never acquire more than two centrioles, suggesting that centriole duplication cannot initiate during G1.

In contrast, the mutant *ts10021*, which arrests in S/G2 after the point at which cells become sensitive to nuclear DNA synthesis inhibitors but before mitosis [35, 37], contains two centrioles per cell when grown at the permissive temperature (Figure 4b), but accumulates increased numbers of centrioles per cell when arrested (Figure 4a,c). More than one round of centriole duplication apparently occurred, because roughly half of these cells contained more than four centrioles. Centriole amplification was prevented by prior G1 arrest induced by incubation in the dark. We conclude that centriole duplication in *Chlamydomonas* initiates during S/G2, just as in animal cells [38, 39].

To test the cell cycle dependence of de novo assembly, double mutants were constructed between *vfl2* and the cell cycle arrest mutants *ts10006*, *ts10009*, and *ts10021*, which arrest in G1, G1, and S/G2, respectively. De novo assembly during arrest should result in a decrease in the fraction of centrioleless cells. As seen in Figure 4d, the fraction of centrioleless cells remains constant after arrest in *vfl2 ts10006* and *vfl2 ts10009*, both of which arrest in G1, but decreases substantially after arrest in *vfl2 ts10021*, which arrests in S/G2. The extent of de novo assembly was greater in *vfl2 ts10021* cells arrested for longer periods (Figure 4e). These results confirm that centrioles can form de novo in *vfl2* cells and indicate that de novo centriole assembly occurs during S/G2.

Measuring de novo centriole assembly using the segregation mutant *vfl3*

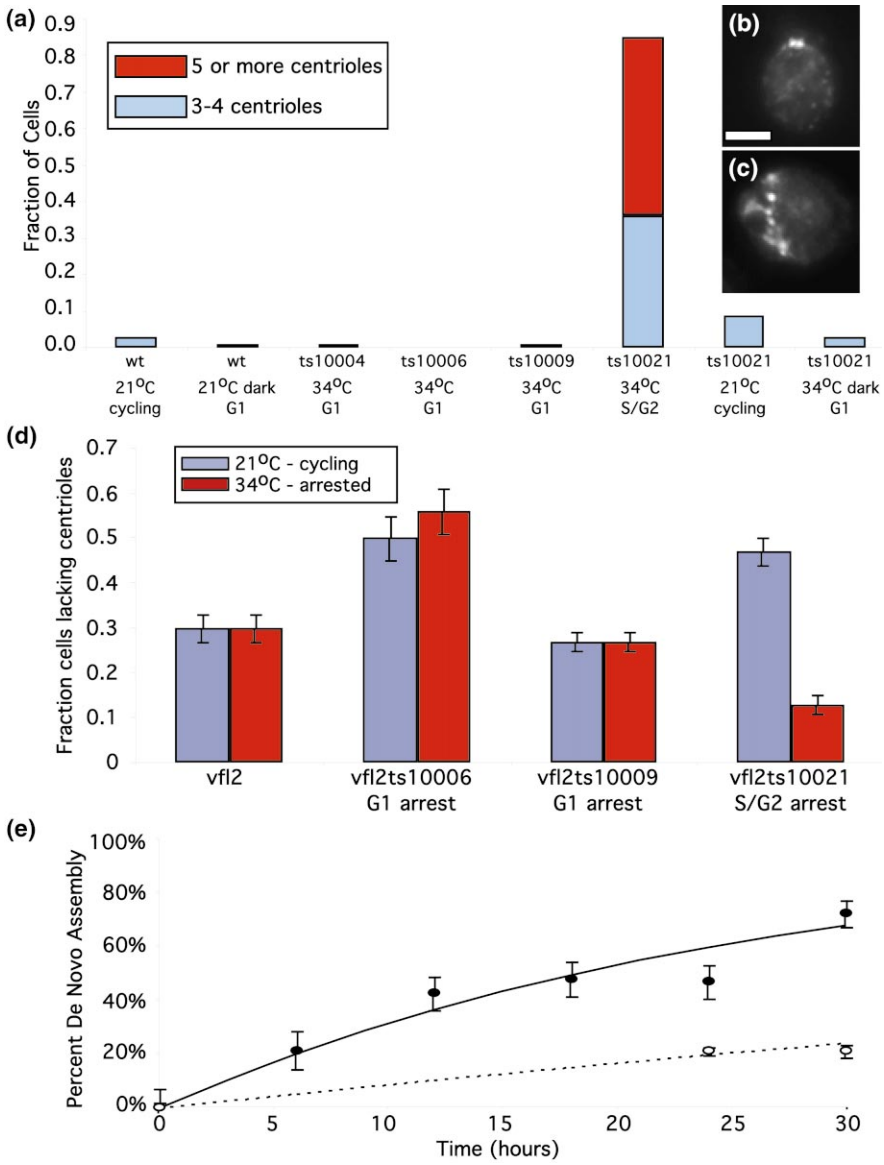
These experiments have all relied on a single mutant, *vfl2*. To rule out the possibility that de novo centriole assembly might be unique to the *vfl2* mutant background, we constructed a double mutant of *ts10021* with a different centriole segregation mutant *vfl3* [33]. Unlike *vfl2* mutants, *vfl3* cells have normal levels of centrin and contain normal nucleus–basal body connectors. Nevertheless, as shown in Figure 4e, centrioles can form de novo in *vfl3* cells arrested in S/G2, confirming that de novo centriole assembly is not just a special feature of *vfl2* mutants.

Role of centrin and VFL3p in the templated centriole assembly pathway

What molecules are involved in centriole duplication? One strong candidate is centrin [40, 41], which has been shown to be necessary for centrosome reproduction [42] and whose yeast homolog, *CDC31*, is required for yeast spindle pole body (SPB) duplication [43]. In yeast, CDC31p assembles into a structure called the half bridge [44] that appears to play a crucial role in nucleating the assembly of a new spindle pole body, and it has recently been proposed that centrin might play an identical role in centriole duplication [45]. To test this proposal, we note that the *vfl2* mutation is caused by a point mutation in the centrin gene [26] that causes the overall levels of centrin protein to be reduced to 25% of wild-type levels, and prevents proper assembly of centrin-based structures [16]. We therefore set out to measure the rate of templated centriole assembly in the *vfl2* mutant background.

In wild-type cells, the number of centrioles doubles with each division as a result of templated centriole duplication. To measure the rate of templated centriole assembly in the *vfl2* mutant background, we examined flagellated cells embedded in agarose pads using the same method as in Figure 3. As shown in Figure 5, biflagellated *vfl2* cells, which initially contain two centrioles, sometimes produce pairs of daughter cells that have less than four flagella between them. This means that fewer than two new centrioles were produced. The data in Figure 5a indicate that after a single generation, the average number of flagella per cell in the progeny of a biflagellated cell is 1.7 ± 0.09 ($n = 22$), which is significantly less than the wild-type value of 2 ($t = -3.33$, $P < 0.0025$), suggesting that each preexisting centriole gives rise, on average, to 0.7 new centrioles per generation, instead of always form-

Figure 4

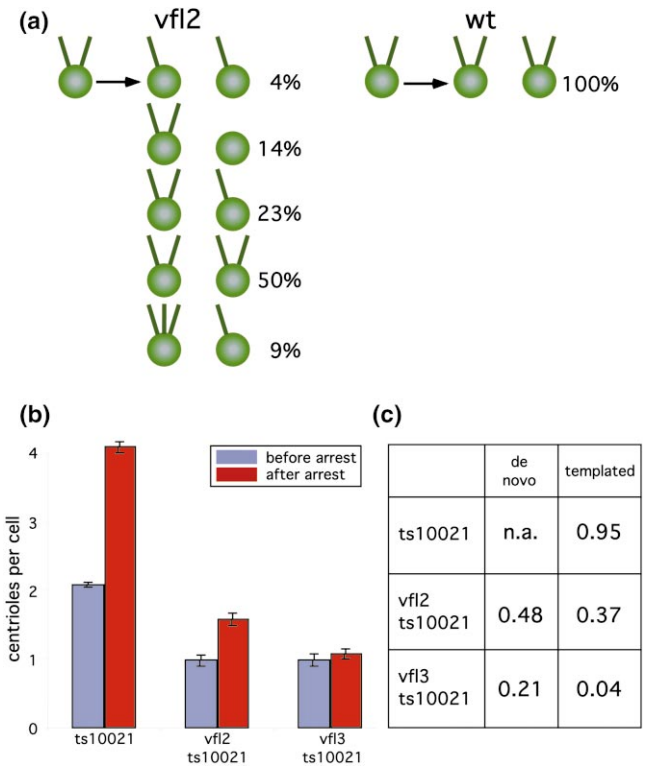


Cell cycle regulation of centriole duplication in *Chlamydomonas*. **(a)** Fraction of cells containing three to four centrioles (indicating a single round of centriole duplication has occurred) or five or more centrioles (indicating multiple rounds of duplication). *ts10021* mutants that arrest in S/G2 appear to undergo multiple rounds of centriole duplication. **(b)** *ts10021* mutants at permissive temperature have two centrioles per cell. **(c)** *ts10021* cells arrested in S/G2 showing supernumerary centrioles based on Fla10 staining. The scale bar represents 5 μm. **(d)** Cell cycle control of de novo centriole assembly. Fraction of cells containing no centrioles plotted for *vfl2* cell cycle arrest double mutants. Only cells arresting in S/G2 show a decrease in the fraction of centrioleless cells, reflecting de novo centriole assembly. **(e)** Kinetics of de novo centriole assembly in S/G2-arrested *vfl2* and *vfl3* cells. De novo assembly in *vfl2 ts10021* cells versus duration of arrest (•). De novo assembly in *vfl3 ts10021* cells versus duration of arrest (o). The ability of centrioles to form de novo in *vfl3* mutants confirms that de novo assembly is not limited to *vfl2* mutant cells.

ing one new centriole as in wild-type cells. In contrast, when wild-type cells were embedded in the identical manner, 100% (n = 12) of biflagellated wild-type cells gave rise to two biflagellated progeny in one generation (Figure 5b). Thus, the *vfl2* mutation appears to cause a partial, but significant, defect in templated centriole duplication.

To verify the templated assembly defect using a different approach, the rate of templated centriole assembly was also measured in S/G2 arrested cells (Figure 5b). *ts120021* cell cycle arrest mutants that start out with 2.1 ± 0.03 (n = 200) centrioles per cell accumulate on average 4.1 ± 0.08 (n = 200) centrioles per cell after arrest, while under the same conditions, *vfl2 ts10021* double mutants starting with 1.0 ± 0.08 (n = 230) centrioles per cell only accumulate 1.6 ± 0.09 (n = 210). But some of these were formed by de novo assembly in centrioleless cells and thus should not be counted when determining templated assembly. Subtracting the number of centrioles that formed de novo during the arrest, calculated from the decrease in the fraction of cells lacking centrioles (Figure 4d), we find that the *vfl2* mutation appears to reduce the templated assembly rate from 0.95 new centrioles per preexisting centriole in *ts10021* cells under these conditions to 0.37 new centrioles per preexisting centriole in *vfl2 ts10021*. This confirms the result from Figure 5a that a mutation in centrin causes a decrease in the templated centriole assembly rate. Because these cells are arrested in S/G2 and are not dividing, this rules out the possibility that

Figure 5



	de novo	templated
ts10021	n.a.	0.95
vfl2 ts10021	0.48	0.37
vfl3 ts10021	0.21	0.04

vfl2 and *vfl3* mutations decrease templated centriole assembly. **(a)** Centriole duplication efficiency in dividing *vfl2* cells. Pedigree analysis of embedded biflagellate *vfl2* and wild-type cells. The total number of flagella doubles each generation in wild-type cells because of normal centriole duplication, but in *vfl2* cells, mutants' flagellar number does not always double, indicating a duplication defect. **(b)** Centriole duplication efficiency in S/G2-arrested cells. The graph plots the average number of centrioles per cell, as judged by FLA10 staining before and after a 24-hr shift to the restrictive temperature. Under conditions where the average number of centrioles doubles in wild-type cells, both *vfl2* and *vfl3* mutants show incomplete duplication. **(c)** Extent of de novo and templated centriole assembly after S/G2 arrest. The templated rate was found by subtracting the number of centrioles formed de novo. The *vfl3* mutant shows an almost complete loss of templated assembly.

the apparent defect in centriole duplication observed in Figure 5a could have been due to the loss of centrioles during cell division. These results suggest that centrin plays a role in templated centriole assembly.

vfl3 mutant cells show an even more dramatic defect in templated centriole assembly. *vfl3 ts10021* cells go from 1.0 ± 0.09 (n = 108) centrioles per cell prior to arrest to 1.1 ± 0.07 (n = 173) centrioles per cell after arrest (Figure 5b). Subtracting the number of centrioles formed per cell by de novo assembly, we find that each preexisting centriole in a *vfl3* mutant gives rise on average to just 0.04 new centrioles under conditions where in normal S/G2-arrested *ts100021* cells each centriole would produce one new centriole (as tabulated in Figure 5c). These data suggest that the VFL3 gene product is required for templated centriole assembly.

Discussion

Alternative explanations for apparent de novo centriole assembly

The result that the centrin defect in *vfl2* mutants causes a partial defect in templated centriole assembly raises an alternate explanation for the de novo centriole assembly reported here. If, when templated duplication fails, an immature procentriole is generated, the subsequent maturation of this procentriole into a visible centriole during the next generation might be mistaken for de novo centriole assembly.

Our data are inconsistent with this model. First, in *vfl2* cells, immature procentrioles are not detected using antibodies to six different centriole-associated proteins, including two (centrin and p210) known to be incorporated into nascent procentrioles at the very earliest detectable stages of centriole assembly [20]. In ferns, centrin localizes to the blepharoplast, the precursor structure that gives rise to de novo basal bodies [23], and in *Naegleria*, centrin localizes to the site of de novo basal body assembly before centriole structures have begun to appear [24]. Thus, the lack of centrin staining in flagellaless *vfl2* cells indicates that they do not contain nascent procentrioles. One can argue, however, that an even earlier precursor is produced in *vfl2* mutants that cannot be detected by any existing antibodies.

A second, stronger argument against the invisible procentriole model is that the duplication defect in *vfl2* mutants is only partial, so that in the majority of flagellated cells, centriole duplication occurs normally as seen in Figure 5a. Moreover, only a minor fraction of the flagellaless cells produced in each generation are the progeny of flagellated cells undergoing an apparent duplication failure. Instead, most nonflagellated *vfl2* cells are the progeny of other nonflagellated cells. A population of *vfl2* cells contains roughly 50% flagellaless, 15% uniflagellate, and 35% biflagellate cells. Pedigree analysis indicates that flagellaless cells produce two flagellaless progeny in 53% of divisions and a single flagellaless progeny in 33% of divisions (Figure 3e). Biflagellate cells produce a single flagellaless progeny in only 14% of divisions (Figure 5a). Uniflagellar cells produce a single flagellaless progeny in 50% of divisions (data not shown). Multiplying the frequency of each cell type in the population by the average number of flagellaless cells it will produce, we find that only 5% of the flagellaless cells formed in any generation could potentially have inherited an invisible procentriole. This number is an order of magnitude too small to account for the fact that almost 50% of flagellaless cells acquire flagella within one generation, and that 100% of flagellaless cells eventually acquire mature functional centrioles.

Therefore, the apparent de novo centriole assembly observed here cannot be due to delayed maturation of invisible procentrioles that are inherited following a duplication failure caused by the *vfl2* mutation.

Implications for the mechanism of centriole duplication

Why do new centrioles only form next to preexisting ones? The most straightforward model is that preexisting centrioles might be required to physically nucleate an assembly of new centrioles, much the same as spindle pole bodies are required to nucleate a new SPB assembly in yeast [45]. In this model, new centrioles only form next to old ones because elsewhere in the cell, de novo centriole assembly would either not occur at all or would occur so slowly that it could not compete with the templated assembly by preexisting centrioles.

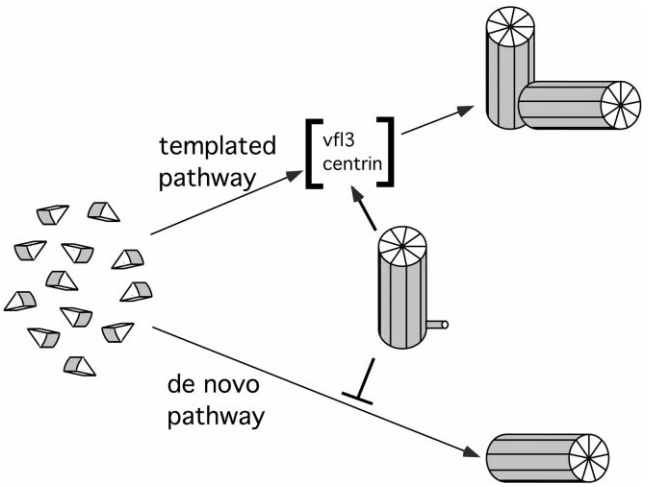
Our data argue that the nucleation of centriole assembly by preexisting centrioles is not by itself sufficient to explain the high fidelity of centriole duplication. We find that in cells lacking centrioles, roughly half will form new centrioles de novo in a single generation. Since a centriole, when present, only forms one new centriole per generation, we conclude that preexisting centrioles only increase the rate of centriole formation by a factor of two. This same 2-fold increase in assembly rate was seen using an entirely different measurement in cells that are arrested in S/G2, where under conditions in which each preexisting centriole produced one new centriole, roughly half of the centrioleless cells acquired new centrioles.

We therefore conclude that preexisting centrioles do not increase the rate of centriole assembly over de novo to the degree required to account for the high fidelity of centriole duplication. Our data would predict that in normal cells with centrioles, half of all cells on average should also form extraneous centrioles by de novo assembly, but this does not in fact happen. We suggest instead (Figure 6) that there must be a negative regulatory mechanism by which centrioles, when present, suppress the de novo assembly pathway.

One potential caveat is that we are measuring the de novo assembly rate in *vfl2* mutants, which have a small but significant defect in templated assembly. Thus, the de novo assembly rate in wild-type cells, if we had a way to measure it, might be even faster. This would only strengthen our conclusion, however, that the de novo assembly rate is too high to account for the fidelity of centriole duplication without invoking a negative regulatory mechanism.

We therefore propose that there are two pathways for centriole assembly, templated and de novo. The templated pathway is activated by the presence of preexisting centrioles, perhaps by a direct nucleating mechanism as proposed for yeast spindle pole bodies, while the de novo pathway is inhibited by preexisting centrioles. In principle, it should be possible for cells to turn off this inhibition in order to generate large numbers of centrioles in parallel by de novo assembly even in the presence of preexisting centrioles, such as has been observed by time-lapse imaging in wasp embryos [46].

Figure 6



Mechanism of centriole duplication. Centrioles can duplicate by either of two pathways, a templated pathway that assembles a new centriole onto a preexisting centriole, or a de novo pathway. The high rate of de novo assembly reported here suggests that the presence of a centriole must negatively regulate the de novo pathway. Both centrin and VFL3 appear to play a role in the templated assembly pathway.

These two pathways are, to some extent, genetically separable, as indicated by the fact that templated assembly is almost completely eliminated in *vfl3* mutants while de novo assembly can still occur in them (Figure 5c). The lack of templated assembly in *vfl3* mutants could explain why centrioles are always unpaired in *vfl3* cells; since new centrioles only form by the de novo pathway, they do not start out attached to any other centrioles. The VFL3 gene has recently been cloned and found to be a novel coiled-coil protein with homologs in mammals (Bode and Silflow, 9th International Conference on Cell and Molecular Biology of *Chlamydomonas*, abstract 151). The *vfl2* mutation, which is a partial loss-of-function mutation in the gene encoding centrin, also seems to show a defect in the templated assembly pathway, which supports the recently discussed model [45] that centrin is part of some nucleating structure similar to the half bridge of the yeast spindle pole body. This nucleating structure may correspond to the polar organizer, a hypothetical structure proposed by Sluder and Rieder [47] to be involved in initiating new spindle pole assembly in higher eukaryotes.

Conclusions

We have measured, for the first time, the rate of de novo centriole assembly in dividing cells that normally contain centrioles. We find that de novo centriole assembly is extremely efficient, and is only 2-fold slower than the normal rate of centriole duplication. These results show that centriole duplication cannot simply be accounted for by the nucleation of new centriole assembly by preexisting centrioles. Instead, there must be a negative regulatory mechanism that inhibits de novo centriole assembly when centrioles are present. This raises the interesting question as to how the cell senses the presence or absence of preexisting centrioles in order to inhibit or activate the de novo assembly pathway.

Materials and methods

Microscopy

Immunofluorescence was performed as described [28]. Antibodies against Fla10 kinesin [28], acetylated tubulin (Sigma Immunochemicals), *Chlamydomonas* centrin, and p210 were used at dilutions of 1:200, 1:10, 1:20, and 1:100, respectively.

The immunofluorescence fixation procedure caused a fraction of flagella to become detached from cells, presumably due to shear forces on the cover slip. For example, wild-type cells showed 11% (n = 110) of cells lacking flagella after immunofluorescence, whereas just 2% (n = 828) of wild-type cells in suspension culture lacked flagella. Likewise, for *vfl2* cells, 81% (n = 141) of cells were flagellaless after immunofluorescence, whereas 49% (n = 500) of *vfl2* cells were flagellaless in suspension culture.

The shearing of flagella during fixation gave rise to apparent exceptions to the general trend that the number of centrioles matched the number of flagella in individual cells as judged by immunofluorescence (Figure 2a,b). Specifically, we observed that 10% of flagellaless *vfl2* cells (n = 115) showed one or more foci of FLA10 staining. The frequency of such cells is consistent with them arising from the loss of flagella from originally flagellated cells during fixation for immunofluorescence. Likewise, 29% of uniflagellate cells (n = 28) showed two centriole foci rather than just one. Again, the frequency of such cells suggests that they arose from originally biflagellated cells that lost one of their two flagella by shearing during fixation. This loss of flagella during fixation necessitated the statistical analysis in Figure 2c, which compares distributions of flagella in cells in suspension culture with the distribution of centrioles in cells fixed for immunofluorescence, thereby avoiding any complications due to flagella shearing during fixation. The graphs in Figure 2c were based on the following numbers of cells: Fla10: 500 cells from suspension culture scored for flagellar number and 291 cells scored for centriole number by immunofluorescence; centrin: 400 cells scored for flagella, 400 cells scored for centrioles; acetyltubulin: 266 cells scored for flagella, 92 scored for centrioles; γ-tubulin: 168 cells scored for flagella, 100 cells scored for centrioles. As discussed in the text, this analysis confirmed the correspondence between centriole number and flagellar number.

For electron microscopy, cells were fixed [48] and processed [49] as previously described, and embedded in Poly/Bed 812 resin. Sections were stained with 2% uranyl acetate (methanolic) and lead citrate, and examined in a Zeiss 10C operated at 80 kV.

Pedigree analysis

For pedigree analysis of cells embedded in 0.1% agarose [14] in TAP media [50], initial flagellar number was noted, and its position was determined using the stage micrometer. After overnight incubation, the flagellar number on progeny cells was recorded. Not all cells divided under these growth conditions, though the survival rates for flagellaless cells versus biflagellate cells (71% and 76%, respectively) were not significantly different ($X^2 = 0.64$, $0.25 < P < 0.5$).

For pedigree analysis of cells in suspension, individual cells were transferred to wells in 96-well microtiter plates by serial dilution. Flagellar number was inferred by analyzing motility with a dissecting microscope. To verify that flagellar number can be reliably determined by motility in *vfl2* cells, we compared the distribution of nonswimming, spinning, and swimming cells in microwells (51%, 16%, and 33%, respectively) to the distribution of cells with zero, one, or two flagella (54%, 13%, and 33%, respectively). These distributions were identical ($X^2 = 0.6$, $0.5 < P < 0.75$).

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